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(54) Multiplex genomic DNA amplification for deletion detection.

(57) The present invention relates to a method for detecting multiple DNA sequences simultaneously. The method involves amplification of multiple sequences simultaneously by annealing a plurality of paired oligonucleotide primers to single stranded DNA. One member of each pair is complimentary to the sense strand of a sequences and the other member is complimentary to a different segment of the anti-sense strand of the same sequence. The amplification occurs by alternately annealing and extending the primers. The invention also includes oligonucleotide primer sequences helpful in detecting genetic diseases and/or exogenous DNA sequences.

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Multiplex Genomic DNA Amplification for Deletion Detection

Field of the Invention

This invention relates to the field of simultaneous detection of deletions in genomic DNA sequences by the process of amplification of multiple sequences within the hemizygous or homozygous genome. The nucleic acid sequences are amplified by the process of simultaneous multiple repetitive reactions. This method of deletion detection is useful in a variety of areas including screening for genetic disease, and animal husbandry. Multiplex DNA amplification is also applicable to the simultaneous analysis of multiple genomic sequences and is useful in forensic medicine, disease screening, and in the development of recombinant or transgenic organisms.

Background

This invention is an improvement on currently established procedures for the detection of genetic diseases resulting from mutations and deletions in genomic DNA sequences. Prenatal diagnosis and carrier detection of many X-linked diseases is available via Southern analysis using full length CDNA clones. Unfortunately, there are several major limitations that prevent widespread and routine use of Southern analysis for diagnosis of genetic disease. In many of the X-linked diseases, the defective sequences are unknown and probes are unavailable. In other diseases, such as X-linked muscular dystrophy, there are multiple exons, at least 60, scattered over a large area of genomic DNA, approximately 2.4 million bases. The introns average 35 Kb in length. In the case of muscular dystrophy, at least 7-9 separate cDNA subclones are necessary for Southern blot analysis to resolve each exon-containing restriction fragment for hyplotype assignment or diagnosis of genomic alterations. Furthermore, Southern analysis is an expensive, tedious, and time-consuming technique that requires the use of radioisotopes, making it unsuitable for routine use in clinical laboratories.

An alternative to Southern analysis for mutation and deletion detection is the polymerase chain reaction (PCR) described by Mullis et al. in U. S. Patent No. 4,683,195 which issued on July 28, 1987 and by Mullis in U. S. Patent No. 4,683,202 which issued on July 28, 1987. With PCR, specific regions of a gene can be amplified up to a million-fold from nanogram quantities of genomic DNA. After amplification the nucleic acid sequences can be analyzed for the presence of mutant alleles either by direct DNA sequencing or by hybridization with allele-specific oligonucleotide probes. The PCR technique has proven useful in the diagnosis of several diseases including β-thalassemia, hemophilia A, sickle cell anemia and phenylketonuria. Routine screening for genetic diseases and exogenous DNA sequences, such as virus, with PCR, has been limited by the ability to conduct tests for only a single sequence at a time. Screening for a plurality of possible DNA sequences requires a cumbersomely large number of separate assays, thus increasing the time, expense, and tedium of performing such assays. For example, in some diseases, such as Duchenne muscular dystrophy (DMD), PCR diagnosis has been limited since point mutations leading to DMD have not been identified. Approximately 60% of the cases of DMD are due to deletions. The other 40% are unknown at present, but probably involve mutations of the intron-exon splice sites or the creation of premature stop codons. Thus a large gene like the DMD gene must be screened with multiple assays.

In both U. S. Patent Nos. 4,683,195 and 4,683,202, procedures are described for amplification of specific sequences. Both patents describe procedures for detecting the presence or absence of at least one specific nucleic acid sequence in a sample containing a mixture of sequences. Although the patents claim at least one sequence and state that multiple sequences can be detected, they do not provide an effective procedure for amplifying multiple sequences at the same time. In the examples, single sequences are amplified or multiple sequences are amplified sequentially. Adding primers for a second sequence is usually possible, but when primers for more than two sequences are added the procedure falls apart. The present application is an improvement on the PCR method and solves the problems encountered when primers for multiple sequences are reacted simultaneously. The present invention describes a procedure for simultaneous amplification of multiple sequences, and the application of this multiplex amplification procedure to detect a plurality of deletions within the same gene or within multiple genes.

The procedures of the present application provide improved methods for the detection of deletions in hemizygous genes on the X and Y chromosomes. The procedures are effective in detecting genetic

diseases caused by deletions on the X or Y chromosome, for example, DMD. They are also effective in detecting homozyous deletions and may be used to simultaneously screen for many possible homozygous or hemizygous deletions as long as parts of the appropriate genetic sequences are known. The procedure for multiplex amplification also enables simultaneous analysis of multiple genetic loci regardless of the presence or absence of deletions.

Summary of the Invention

An object of the present invention is a method for simultaneously detecting deletions at a plurality of genomic DNA sequences.

An additional object of the present invention is to detect X-linked genetic diseases.

A further object of the present invention is the diagnosis of DMD.

A further object of the present invention is to simultaneously analyze multiple genetic loci for polymorphisms and/or non-deletion mutations.

Thus, in accomplishing the foregoing objects there is provided in accordance with one aspect of the present invention, a method for simultaneously detecting deletions at a plurality of genomic DNA sequences, comprising the steps of:

Treating said genomic DNA to form single stranded complementary strands;

Adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;

Annealing the plurality of primers to their complementary sequences;

Simultaneously extending said plurality of annealed primers from each primer's 3′ terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separating from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;

Separating said extension products from said templates to produce single-stranded molecules;

Amplifying said single stranded molecules by repeating at least once, said annealing, extending and separating steps; and

Identifying said amplified extension products from each different sequence.

Additional embodiments include detection of deletions at a plurality of genomic DNA sequences on the X and Y chromosomes or on autosomal chromosomes when the deletions are homozygous. A variety of X-linked diseases can be detected including ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferfase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.

In another embodiment, X-linked muscular dystrophy is detected using a plurality of paired primers which are complementary to different sequences within the gene coding for the protein dystrophin. Other embodiments include multiple oligonucleotide primers useful in detecting X-linked genetic disease.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure when taken in conjunction with the accompanying drawings.

Brief Discussion of the Drawings

The invention will be more readily understood from a reading of the following specification and by references to the accompanying drawings, forming a part thereof:

Figure 1 is a schematic representation of the DMD gene illustrating the approximate size of the locus, the position of the amplified fragments and the location of the genomic regions that have been cloned and sequenced.

Figure 2 is an example of a PCR reaction used to detect a deletion in fetal DNA for prenatal diagnosis.

Figure 3 represents the multiplex DNA amplification of lymphoblast DNA from unrelated male DMD patients. A. and B. show two sets of ten samples. Each DRL # refers to the R.J. Kleberg Center for Human Genetics Diagnostic Research Laboratory family number. MW: Hae III digested ϕX174 DNA. (-): no template

DNA added to the reaction. The relationship between the amplified region and the region on the gene is indicated to the right of A. The letters correspond to those on Figure 1.

Figure 4 represents Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from an affected male (AM; lymphoblast DNA) and a male fetus (MF; cultured amniotic fluid cell DNA) from six different families. Both the affected male and the fetal DNAs of DRL #s 521 and 531 display a deletion of region f (Fig. 1) diagnosing these fetuses as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL # 483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique.

Figure 5 represents Multiplex DNA amplification from chorionic villus specimen (CVS) DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1), diagnosing the fetus as affected. CVS DNA from DRL # 120 did not display a deletion with this technique.

Figure 6 shows amplification of seven exon regions of the DMD locus.

The drawings are not necessarily to scale and certain features of the invention may be exaggerated in scale or shown in schematic form in the interests of clarity and conciseness.

Detailed Description

It will be readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein, without departing from the scope and spirit of the invention.

The term "oligonucleotide primers" as used herein defines a molecule comprised of more than three deoxyribonucleotides or ribonucleotides. Its exact length will depend on many factors relating to the ultimate function and use of the oligonucleotide primer, including temperature, source of the primer and use of the method. The oligonucleotide primer can occur naturally, as in a purified restriction digest, or be produced synthetically. The oligonucleotide primer is capable of acting as an initiation point for synthesis when placed under conditions which induce synthesis of a primer extension product complementary to a nucleic acid strand. The conditions can include the presence of nucleotides and an inducing agent such as a DNA polymerase at a suitable temperature and pH. In the preferred embodiment, the primer is a single-stranded oligodeoxyribonucleotide of sufficient length to prime the synthesis of an extension product from a specific sequence in the presence of an inducing agent. In the deletion detection procedure, the oligonucleotides are usually at least greater than 12 mers in length. In the preferred embodiment, the oligonucleotide primers are about 18 to 29 mers in length. Sensitivity and specificity of the oligonucleotide primers are determined by the primer length and uniqueness of sequence within a given sample of template DNA. Primers which are too short, for example, less than about 12 mers may show non-specific binding to a wide variety of sequences in the genomic DNA and thus are not very helpful. In the preferred embodiment, the oligonucleotide primer is usually selected for its ability to anneal to intron sequences in the proximity of the 5′ or 3′ end of the exon or to anneal to a sequence at the intron-exon junction. Since the known deletion defects resulting in genetic diseases result from deletions that include the exons or intron-splice site regions, it is preferable to have primers complementary to intron sequences.

Each primer pair herein was selected to be substantially complementary to the different strands of each specific sequence to be amplified. Thus, one primer of each pair is sufficiently complementary to hybridize with a part of the sequence in the sense strand and the other primer of each pair is sufficiently complementary to hybridize with a different part of the same sequence in the anti-sense strand. Thus, although the primer sequence need not reflect the exact sequence of the template, the more closely it does reflect the exact sequence the better the binding during the annealing stage.

Within a primer pair, each primer preferably binds at a site on the sequence of interest distant from the other primer. In the preferred embodiment the distance between the primers should be sufficient to allow the synthesis of an extension product between the two binding sites, yet close enough so that the extension product of each primer, when separated from its template, can serve as a template for the other primer. The extension products from the two paired primers are complementary to each other and can serve as templates for further synthesis. The further apart the binding sites, the more genomic DNA which can be screened. However, if the distance is too great the extension products will not efficiently overlap with the primers and thus amplification will not occur.

As used herein the term "extension product" refers to the nucleotide sequence which is synthesized from the 3′ end of the oligonucleotide primer and which is complementary to the strand to which the

oligonucleotide primer is bound.

As used herein the term "differentially labeled" shall indicate that each extension product can be distinguished from all the others because it has a different label attached or is of a different size or binds to a specifically labelled oligonucleotide. One skilled in the art will recognize that a variety of labels are available. For example, these can include radioisotopes, fluorescers, chemiluminescers, enzymes and antibodies. Various factors affect the choice of the label. These include the effect of the label on the rate of hybridization and binding of the primer to the DNA, the sensitivity of the label, the ease of making the labeled primer, probe or extension products, the ability to automate, available instrumentation, convenience and the like. For example, a different radioisotope could be used such as ^{32}P, ^{3}H, or ^{14}C; a different fluorescer such as fluorescin, tetramethylrhodamine, Texas Red or 4-chloro-7- nitrobenzo-2-oxa-1-diazole (NBD); or a mixture of different labels such as radioisotopes, fluorescers and chemiluminescers. Alternatively, the primers can be selected such that the amplified extension products for each sequence are of different lengths and thus can be separated by a variety of methods known in the art. Similarily, the extension products could include a restriction fragment length polymorphism which could be used to distinguish different extension products. In these examples, each primer or its extension product can be differentiated from all the other primers when they are in a mixture. Alternatively, probes which bind to the amplified extension products could be labeled and sets of probes which distinguish alleles of a single sequence within a multiplex DNA amplification reaction may be used whether or not labelled.

Each specific, different DNA sequence, which is to be detected herein, can derive from genomic DNA of the organism or exogenous DNA such as virus, bacteria or parasites. The source of genomic DNA from the organism to be tested can be blood, hair or tissue (including chorionic villi, amniotic cells, fibroblasts and biopsies). The source of DNA may be freshly obtained or have been suitably stored for extended periods of time. The DNA must be of sufficient quality to permit amplification. The genomic DNA can be prepared by a variety of techniques known to one skilled in the art.

As used herein, the term "deletion" refers to those genomic DNA sequences in which one or more nucleic acid base has been deleted from the sequence and thus is no longer present in the gene. The size of the deletion can affect the sensitivity of the amplification procedure. Generally, the larger the deletion the larger the sensitivity.

Any specific known nucleic acid sequence can be detected by the present method. Preferably, at least part of the sequence is deleted from the genome. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail to prepare oligonucleotide primers which will hybridize to the different strands of the desired sequence at relative positions along the sequence.

The oligonucleotide primers may be prepared using any suitable method, for example, phosphyltriester and phosphyldiester methods or automated embodiments thereof, the synthesis of oligonucleotides on a modified solid support, the isolation from a biological source (restriction endonuclease digestion), and the generation by enzymatically directed copying of a DNA or RNA template.

One embodiment of the present invention is a method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of: treating said DNA to form single stranded complimentary strands; adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complimentary to a part of the sequence in the sense-strand and the other primer of each pair substantially complimentary to a different part of the same sequence in the complimentary anti-sense strand; annealing the plurality of primers to their complimentary sequences; simultaneously extending said plurality of annealed primers from each primer's 3′ terminus to synthesize an extension product complimentary to the strands annealed to each primer, said extension products, after separation from the complement, serving as templates for the synthesis of an extension product from the other primer of each pair; separating said extension products from said templates to produce single-stranded molecules; amplifying said single-stranded molecules by repeating, at least once, said annealing, extending and separating steps; and identifying said amplified extension product from each different sequence.

One preferred embodiment of the present invention is a method for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from a group of sequences on the X and Y chromosomes. It is preferrable to detect hemizygous genes on the X and Y chromosomes, since this increases the level of sensitivity. When the procedure is used to detect the heterozygous state, it requires quantitative measurement, and thus is much less efficient than detecting the presence or absence of sequences as is done for hemizygous genes. For example, if part of an exon has been deleted the multiplex amplification method of the present invention will detect this by either failing to produce an oligonucleotide sequence or by production of an oligonucleotide sequence of a different size. Furthermore multiple exons can be screened at the same time. Thus, it is easy to detect the presence of a deletion.

However, in looking at heterozygous states, where the chromosomes have one normal gene and one deleted gene, the normal gene will produce a normal product, and thus there is the necessity to measure the quantitative difference in the production of extension products.

A second embodiment of the present invention is to permit simultaneous amplification of multiple, possibly unrelated sequences for the purpose of their simulataneous analysis. Such analysis may simply involve the determination of whether exogenous sequences (virus, bacteria or other parasites) are present within a sample of DNA, or might involve the detection of polymorphisms or mutations within a plurality of sequences. The polymorphisms or mutations can be detected by a variety of methods well known to those skilled in the art. The methods include, but are not limited to, direct DNA sequencing, allele-specific oligonucleotide hybridization, and competitive oligonucleotide priming.

The multiplex genomic DNA amplication method is preferably used to detect X-linked diseases resulting from deletions in the genomic DNA sequence. Genetic diseases can be caused by a variety of mechanisms including mutations and deletions. The procedure described herein was developed for detection of genetic diseases which result from deletions within the genome. Examples of some X-linked diseases which are candidates for the use of multiplex genomic DNA amplification are ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy. Other disorders on the X chromosome or genes on the Y chromosome can also be easily detected. The procedure is also applicable to the detection of any set of known point mutations within a set of genomic sequences. The procedure is also applicable to the simultaneous detection of any set of exogenous DNA sequences in a given DNA sample. The procedure is also applicable to the simultaneous detection of any set of polymorphic or variable tandemly repetitive sequences within a genone.

The advantages of the multiplex amplification system are that numerous diseases or specific DNA sequence alterations can be detected in the same assay. For example, primers to hypoxanthine phosphoribosyltransferfase deficiency, steroid sulfatase deficiency, X-linked muscular dystrophy, ornithine transcarbamylase deficiency and other X-linked diseases can all be run simultaneously on the same sample. Furthermore, the multiplex amplification procedure is useful for very large genes with multiple exons, such as the dystrophin gene. Because of the large size of the dystrophin locus, Mullis type PCR amplification is not able to scan the whole gene in one assay. Thus, it is necessary for multiple site amplification within the gene to detect all possible deletions which could result in disease. Deletions at the DMD locus can encompass any of the approximately 60 plus exons which are distributed over more than 2 million bases of DNA. Virtually all of these exons are separated by large introns and so up to 60 separate reactions could be required for complete analysis of DMD deletions. To simplify this task, the present invention of a multiplex genomic DNA amplification for deletion detection can be employed to perform simultaneous examination of multiple exons. For example, oligonucleotide primers flanking separate DMD gene exons can be synthesized and combined and used for multiplex DNA applications. At present, up to at least 7 different DMD gene sequences have been examined simultaneously. The entire procedure for the multiplex amplification from start-up to photography of the results takes less than 5 hours. The relative locations of the amplified regions do not affect the results and exons have been amplified which have been separated by at least 1000 kb. The PCR amplification technique of Mullis is adequate for one and possibly two pair of primers, but when greater than two pairs of primers are used the procedure will not adequately amplify all the appropriate sequences.

One skilled in the art readily appreciates that as more exon gene sequences become available the applicability of this test will expand to examine for deletions in multiple genes at the same time or examine multiple sites within the same gene at the same time. The later example is important for genes such as dystrophin which are so large that primers annealed to the ends of the gene will not traverse the whole gene sequence. Thus the necessity of doing multiple analysis to detect deletions in different regions of the gene. In addition, as specific mutations within multiple unrelated genes become known, multiplex DNA amplification can be applied to simultaneously assay for the presence of any of these mutations.

Furthermore, as specific or highly variable DNA sequence polymorphisms become known in various genetic Loci, multiplex DNA amplification can be used to simultaneously analyze these polymorphisms to determine the haplotype or to determine the identity or source of DNA (genetic footprinting).

The number of analyses which can be run simultaneously is unlimited, however, the upper limit is probably about 20 and is dependent on the size differences required for resolution and/or the number of labels or methods which are available to resolve the extension products. The ability to simultaneously amplify only 9 exons would allow the detection of greater than 90% of all known DMD deletions in a single reaction. The ability to simultaneously amplify even as few as 10 exons allows the rapid and simple diagnosis of DMD deletions using only a few separate reactions. Assuming that there are about 60 exons in the DMD gene and that the exons are widely separated such that primers are needed for every exon, a

maximum of 6 separate assays is needed to detect all deletions in this gene. Under the same assumptions the Mullis PCR method would require 60 separate reactions to detect the deletions in this gene. Thus, as the size of the gene increases and the number of exons which cannot be detected together increases the advantages of this method are greatly enhanced. Furthermore, use of an automatic PCR apparatus (such as that produced by Perkin-Elmer/Cetus) and DNA sequencing machines will facilitate resolution and detection of amplified DNA fragments, will help automate the assay and will lead to the method being applied routinely in clinical laboratories without the need for highly trained research personnel.

The following examples are offered by way of illustration and are not intended to limit the invention in any manner. In the examples all percentages are by weight, if for solids and by volume if for liquids, and all temperatures are in degrees Celsius unless otherwise noted.

EXAMPLE 1

The following conditions are currently in use to perform simultaneous amplification of a plurality of separate genomic regions within the human DMD gene. These conditions may need to be slightly modified depending on the particular regions to be amplified, the number and length of sequences to be amplified, and the choice of oligonucleotide primers. The time of reaction is highly dependent on the overall sequence length. Thus, as the number of amplified sequences increase and/or the length of amplified sequences increases, the time must be increased. The temperature is dependent on the length, the uniqueness of the primer sequence and the relative percentage of GC bases. The longer the primers, the higher the temperature needed. The more unique the sequence, the lower the temperature needed to amplify. GC rich primers need higher temperatures to prevent cross hybridization and to allow unique amplification. However, as the AT percentage increases, higher temperatures cause these primers to melt. Thus, these primers must be lengthened for the reaction to work.

Template DNA was prepared from the tissue chosen for analysis using a variety of well-established methods known to those skilled in the art. Typically, 100 µl reaction volumes were utilized. Approximately 500 ng of DNA was added to a solution comprised of the following: 67 mM Tris-HCL [pH 8.8 at 25°]; 6.7 mM magnesium chloride; 16.6 mM ammonium sulfate; 10 mM β-mercaptoethanol; 6.7 µM ethylene diamine tetra-acetic acid (EDTA); and 170 µg/mL bovine serum albumin. This solution can be prepared beforehand and appears to be stable for very long periods of storage at -70°. The enzyme, Tag polymerase, was added to achieve a final concentration of 100 units/mL. This reaction mixture was gently mixed. The reaction mixture was overlaid with about 50 µL of paraffin oil, and the reaction vessel (preferably a 0.5 ml microcentrifuge tube) was centrifuged at 14,000 x g for 10 sec. Amplification was performed either by manually transferring the reaction vessels between glycerol filled heat blocks at the appropriate temperatures, or automatically transferring the reaction vessels with a Perkin-Elmer/Cetus corporation thermocycler using the 'step-cycle' functions. The reaction was controlled by regulated and repetitive temperature changes of various duration. Initially the reaction was heated to 94° for 7 minutes. Subsequently 25 cycles of the following temperature durations were applied: 94° for 1 minute, then 55° for 45 seconds, then 65° for 3 1/2 minutes. Following completion of the final cycle the reaction was incubated at 65° for an additional 7 minutes. Reactions were then stored at 4° until analysis.

Genomic DNA deletions and/or exogenous DNA sequences were determined by examining the amplification products. For example, the lack of an expected amplification product indicates a deletion. Many methods for this determination are known to those skilled in the art. The preferred method involves electrophoresis of about one-twentieth of the reaction on a 1.4% (weight/vol) agarose gel in the following buffer: 40 mM tris-HCl; 20 mM sodium acetate, 1 mM EDTA (adjusted to pH 7.2 with glacial acetic acid), and 0.5µg/µl. of ethidium bromide. Electrophoresis was performed at 3.7 volts/cM for 100 minutes per 14 cM of agarose gel length. Analysis was completed by examining the electrophoresed reaction products on an ultraviolet radiation transilluminator, and the results were photographed for permanent records.

When the analysis requires determination of DNA sequence polymorphisms or mutations within individual amplification products the agarose gel is transferred to an appropriate DNA binding medium such as nitrocellulose using well-established procedures, for example, Southern blotting. Individual DNA sequences within the amplified DNA fragments can be determined by a variety of techniques including allele-specific oligonucleotide hybridization. Alternatively, reaction products may be further analyzed prior to electrophoresis on agarose gel by competitive oligonucleotide primer amplification, using separate allele-specific primers for each amplified DNA sequence of the multiplex amplification reaction products.

A third method for determining DNA sequence differences within individual amplification products does

not require electrophoresis. In this method, aliquots of the multiplex amplification reaction are sequentially applied to an appropriate DNA binding membrane such as nitrocellulose, and then each aliquot is analyzed via hybridization with individual members of sets of allele-specific oligonucleotide (ASO) probes, each separate aliquot being hybridized with one member of a pair of ASO probes specific for one member of the multiply amplified DNA sequences.

EXAMPLE 2

Figure 1 is a schematic representation of the DMD locus. The relative location of the exons used in the DMD gene amplification examples are illustrated.

For detection of DMD, a variety of probes can be used either in individual PCR reactions or in combinations in multiplex PCR reactions. These probes are shown in Table 1.

Table 1

Summary of DMD gene multiplex amplification primer sets.				
	Exon and Size	Primer Sequence	Amplified	Deleted
a.	Exon 8 (182bp)	F-GTCCTTTACACACTTTACCTGTTGAG R-GGCCTCATTCTCATGTTCTAATTAG	360 bp	11.3%
b.	Exon 17 (178bp)	F-GACTTTCGATGTTGAGATTACTTTCCC R-AAGCTTGAGATGCTCTCACCTTTTCC	416 bp	9.4%
c.	Exon 19 (88bp)	F-TTCTACCACATCCCATTTTCTTCCA R-GATGGCAAAAGTGTTGAGAAAAAGTC	459 bp	10.3%
d.	4.1Kb Hind III (148bp)	F-CTTGATCCATATGCTTTTACCTGCA R-TCCATCACCCTTCAGAACCTGATCT	268 bp	4.0%
e.	0.5Kb Hind III (176bp)	F-AAACATGGAACATCCTTGTGGGGAC R-CATTCCTATTAGATCTGTCGCCCTAC	547 bp	8.4%
f.	1.2/3.8Kb Hind III (159bp)	F-TTGAATACATTGGTTAAATCCCAACATG R-CCTGAATAAAGTCTTCCTTACCACAC	506 bp	18.2%
g.	Exon 12 (151bp)	F-GATAGTGGGCTTTACTTACATCCTTC R-GAAAGCACGCAACATAAGATACACCT	337 bp	9.6%
			Total:	38%

In Table 1 each exon is designated a, b, c, d, e, f, or g and corresponds to the same letter in Fig. 1. When the exon number is known it is listed. If the exon number is not known, the size of the genomic Hind III fragment containing that exon is listed. Also shown is the size of the exon in base pairs (bp). The PCR primer sequences are shown in 5′-3′ orientation. The forward primer (F), hybridizes 5′ of the exon, and the reverse primer (R), hybridizes 3′ of the exon. The size of the amplified fragment obtained with each primer set is also shown.

The percentage of analyzed DMD patients that are deleted for each indicated exon is shown in column four. This total number is less than the sum of the individual exon deletion frequencies because many deletions encompass multiple exons.

In Table 2 are the exon and flanking intron sequences for Exon 17. The exon is from 227 to 402. The primer sequences used to amplify this sequence are 7 to 33 and 396 to 421.

TABLE 2

5' 10	20	30	40	50
TAAATTGACT	TTCGATGTTG	AGATTACTTT	CCCTTGCTAT	TTCAGTGAAC
60	70	80	90	100
CAAACTTAAG	TCAGATAAAA	CAATTTTATT	TGGCTTCAAT	ATGGTGCTAT
110	120	130	140	150
TTTGATCTGA	AGGTCAATCT	ACCAACAAGC	AAGAACAGTT	TCTCATTATT
160	170	180	190	200
TTCCTTTGCC	ACTCCAAGCA	GTCTTTACTG	AAGTCTTTCG	AGCAATGTCT
210	220	230	240	250
GACCTCTGTT	TCAATACTTC	TCACAGATTT	CACAGGCTGT	CACCACCACT
260	270	280	290	300
CAGCCATCAC	TAACACAGAC	AACTGTAATG	GAAACAGTAA	CTACGGTGAC
310	320	330	340	350
CACAAGGGAA	CAGATCCTGG	TAAAGCATGC	TCAAGAGGAA	CTTCCACCAC
360	370	380	390	400
CACCTCCCCA	AAAGAAGAGG	CAGATTACTG	TGGATTCTGA	AATTAGGAAA
410	420	430	440	450
AGCTGAGAGC	ATCTCAAGCT	TTTATCTGCA	AATGAAGTGG	AGAAAACTCA
460	470	480	490	500
TTTACAGCAG	TTTTGTTGGT	GGTGTTTTCA	CTTCAGCAAT	ATTTCCAGAA
510	520	530	540	550
TCCTCGGGTA	CCTGTAATGT	CAGTTAATGT	AGTGAGAAAA	ATTATGAAGT
560	570	580	590	600
ACATTTTAAA	ACTTTCACAA	GAAATCACTA	TCGCAACAGA	AACTAAATGC
610	620	630	640	650
TTAATGGAAA	TGGTGTTTTC	TGGGGTGAAA	GAAGAAACTA	TAGAAACTAT
660	670	680	690	700
AGGTGATAAA	CTACTGTGGT	AGCATTTTAA	TCCTAAAAGT	TTCTTTCTTT
710	720	730	740	750
CTTTTTTTTT	TTTCTTCCTT	ATAAAGGGCC	TGCTTGTTGA	GTCCCTAGTT
760	770	780	790	800
TTGCATTAAA	TGTCTTTTTT	TTCCAGTAAC	GGAAAGTGCA	TTTTCATGAA
810	820	830	840	850
GAAGTACACC	TATAATAGAT	GGGATCCATC	CTGGTAGTTT	ACGAGAACAT
860	870	880	890	900
GATGTCTCAG	TCTGCGCATC	CTAAATCAGG	AGTAATTACA	GAACACATTT
910	920	930	940	950
CCTGTTCTTT	GATATTTATA	AAGTCTTATC	TTGAAGGTGT	TAGAATTTTT
960	970	980	990	1000
AACTGATCTT	TTTGTGACTA	TTCAGAATTA	TGCATTTTAG	ATAAGATTAG
1010	1020	1030	1040	
GTATTATGTA	AATCAGTGGA	TATATTAAAT	GATGGCAATA	A-3'

In Table 3 is the exon and flanking intron sequences for Exon d of Table 1 [or, the exon located on a 4.1 kb Hind III fragment]. The exon is from 295 to 442. The primer sequences used to amplify this sequence are 269 to 293 and 512 to 538.

TABLE 3

5' 10	20	30	40	50
TGTCCAAAAT	AGTTGACTTT	CTTTCTTTAA	TCAATAAATA	TATTACTTTA
60	70	80	90	100
AAGGGAAAAA	TTGCAACCTT	CCATTTAAAA	TCAGCTTTAT	ATTGAGTATT
110	120	130	140	150
TTTTTAAAAT	GTTGTGTGTA	CATGCTAGGT	GTGTATATTA	ATTTTTATTT
160	170	180	190	200
GTTACTTGAA	ACTAAACTCT	GCAAATGCAG	GAAACTATCA	GAGTGATATC
210	220	230	240	250
TTTGTCAGTA	TAACCAAAAA	ATATACGCTA	TATCTCTATA	ATCTGTTTTA
260	270	280	290	300
CATAATCCAT	CTATTTTTCT	TGATCCATAT	GCTTTTACCT	GCAGGCGATT
310	320	330	340	350
TGACAGATCT	GTTGAGAAAT	GGCGGCGTTT	TCATTATGAT	ATAAAGATAT
360	370	380	390	400
TTAATCAGTG	GCTAACAGAA	GCTGAACAGT	TTCTCAGAAA	GACACAAATT
410	420	430	440	450
CCTGAGAATT	GGGAACATGC	TAAATACAAA	TGGTATCTTA	AGGTAAGTCT
460	470	480	490	500
TTGATTTGTT	TTTTCGAAAT	TGTATTTATC	TTCAGCACAT	CTGGACTCTT
510	520	530	540	550
TAACTTCTTA	AAGATCAGGT	TCTGAAGGGT	GATGGAAATT	ACTTTTGACT
560	570	580		
GTTGTTGTCA	TCATTATATT	ACTAGAAAGA	AAA-3'	

In Table 4 is the exon and flanking intron sequences for Exon e Table 1 [0.5 Kb Hind III fragment exon]. The exon is from 398 to 571. The primer sequences used to amplify this sequence are 51 to 75 and 572 to 597.

TABLE 4

5' 10	20	30	40	50
ACCCAAATAC	TTTGTTCATG	TTTAAATTTT	ACAACATTTC	ATAGACTATT
60	70	80	90	100
AAACATGGAA	CATCCTTGTG	GGGACAAGAA	ATCGAATTTG	CTCTTGAAAA
110	120	130	140	150
GGTTTCCAAC	TAATTGATTT	GTAGGACATT	ATAACATCCT	CTAGCTGACA
160	170	180	190	200
AGCTTACAAA	AATAAAAACT	GGAGCTAACC	GAGAGGGTGC	TTTTTTCCCT
210	220	230	240	250
GACACATAAA	AGGTGTCTTT	CTGTCTTGTA	TCCTTTGGAT	ATGGGCATGT
260	270	280	290	300
CAGTTTCATA	GGGAAATTTT	CACATGGAGC	TTTTGTATTT	CTTTCTTTGC
310	320	330	340	350
CAGTACAACT	GCATGTGGTA	GCACACTGTT	TAATCTTTTC	TCAAATAAAA
360	370	380	390	400
AGACATGGGG	CTTCATTTTT	GTTTTGCCTT	TTTGGTATCT	TACAGGAACT
410	420	430	440	450
CCAGGATGGC	ATTGGGCAGC	GGCAAACTGT	TGTCAGAACA	TTGAATGCAA
460	470	480	490	500
CTGGGGAAGA	AATAATTCAG	CAATCCTCAA	AAACAGATGC	CAGTATTCTA
510	520	530	540	550
CAGGAAAAAT	TGGGAAGCCT	GAATCTGCGG	TGGCAGGAGG	TCTGCAAACA
560	570	580	590	600
GCTGTCAGAC	AGAAAAAAGA	GGTAGGGCGA	CAGATCTAAT	AGGAATGAAA
610	620			
ACATTTTAGC	AGACTTTTTA	AGCTT-3'		

In Table 5 is the exon and flanking intron sequences for Exon f, Table 1 [overlaps the 1.2 Kb and 3.8 Kb Hind III fragments]. The exon is from 221 to 406. The primer sequences used to amplify this sequence are 26 to 53 and 516 to 541.

TABLE 5

5' 10	20	30	40	50
TTTTGTAGAC	GGTTAATGAA	TAATTTTGAA	TACATTGGTT	AAATCCCAAC
60	70	80	90	100
ATGTAATATA	TGTAAATAAT	CAATATTATG	CTGCTAAAAT	AACACAAATC

110	120	130	140	150
AGTAAGATTC	TGTAATATTT	CATGATAAAT	AACTTTTGAA	AATATATTTT
160	170	180	190	200
TAAACATTTT	GCTTATGCCT	TGAGAATTAT	TTACCTTTTT	AAAATGTATT
210	220	230	240	250
TTCCTTTCAG	GTTTCCAGAG	CTTTACCTGA	GAAACAAGGA	GAAATTGAAG
260	270	280	290	300
CTCAAATAAA	AGACCTTGGG	CAGCTTGAAA	AAAAGCTTGA	AGACCTTGAA
310	320	330	340	350
GAGCAGTTAA	ATCATCTGCT	GCTGTGGTTA	TCTCCTATTA	GGAATCAGTT
360	370	380	390	400
GGAAATTTAT	AACCAACCAA	ACCAAGAAGG	ACCATTTGAC	GTTAAGGTAG
410	420	430	440	450
GGGAACTTTT	TGCTTTAATA	TTTTTGTCTT	TTTTAAGAAA	AATGGCAATA
460	470	480	490	500
TCACTGAATT	TTCTCATTTG	GTATCATTAT	TAAAGACAAA	ATATTACTTG
510	520	530	540	550
TTAAAGTGTG	GTAAGGAAGA	CTTTATTCAG	GATAACCACA	ATAGGCACAG
560	570	580	590	600
GGACCACTGC	AATGGAGTAT	TACAGGAGGT	TGGATAGAGA	GAGATTGGGC
610	620	630	640	650
TCAACTCTAA	ATACAGCACA	GTGGAAGTAG	GAATTTATAG	C-3'

In Table 6 is the exon and flanking intron sequences for Exon 12. The exon is from 1[illegible] to 329. The primer sequences used to amplify this sequence are 27 to 52 and 332 to 357.

TABLE 6

5' 10	20	30	40	50
TGAGAAATAA	TAGTTCCGGG	GTGACTGATA	GTGGGCTTTA	CTTACATCCT
60	70	80	90	100
TCTCAATGTC	CAATAGATGC	CCCCAAATGC	GAACATTCCA	TATATTATAA
110	120	130	140	150
ATTCTATTGT	TTTACATTGT	GATGTTCAGT	AATAAGTTGC	TTTCAAAGAG
160	170	180	190	200
GTCATAATAG	GCTTCTTTCA	AATTTTCAGT	TTACATAGAG	TTTTAATGGA
210	220	230	240	250
TCTCCAGAAT	CAGAAACTGA	AAGAGTTGAA	TGACTGGCTA	ACAAAACAGA
260	270	280	290	300
AGAAAGAACA	AGGAAAATGG	AGGAAGAGCC	TCTTGGACCT	GATCTTGAAG
310	320	330	340	350
ACCTAAAACG	CCAAGTACAA	CAACATAAGG	TAGGTGTATC	TTATGTTGCG
360	370	380	390	400
TGCTTTCTAC	TAGAAAGCAA	ACTCTGTGTA	TAGTACCTAT	ACACAGTAAC
410	420	430	440	450
ACAGATGACA	TGGTTGATGG	GAGAGAATTA	AAACTTAAAG	TCAGCCATAT
460	470	480	490	500
TTTAAAAATT	ATTTTTACCT	AATTGTTTTT	GCAATCTTTG	TTGCCAATGG
510	520	530	540	550
CCTTGAATAA	GTCCCCTCCA	AAATTCAGGT	GATTGTATTA	GGAGATGGAA

```
       560        570        580        590        600
TATTTAAGGG TGAATAATCC ATCAGGGCTC CTCCCTTAAG AATAGGATCA
       610        620        630        640        650
AGTCCCATAT AAAAGAGGCT TCACACAGTG TTCTCCTATC TCTTGACCCT
       660        670        680        690        700
CCACCATGCA CCACCATGTG AAAACTCTGT GAAAAGGCCC TCACCAGATG
       710        720        730        740        750
CTAACATCTT GATCTTGGAT TTCCCAAACT CGAGAACTGT GAAAAAATAA
       760        770        780        790        800
AGGTACATTC TTCCTAAATT ACCTCATTCT CATTTAAACA CACAAAGTGC
       810
ACACATAGCT G-3'
```

In Table 7 is the exon and flanking intron sequences for the Exon located on a 10 Kb Hind III fragment. The exon is from 1 to 150.

TABLE 7

```
5'      10         20         30         40         50
TTACTGGTGG AAGAGTTGCC CCTGCGCCAG GGAATTCTCA AACAATTAAA
        60         70         80         90        100
TGAAACTGGA GGACCCGTGC TTGTAAGTGC TCCCATAAGC CCAGAAGAGC
       110        120        130        140        150
AAGATAAACT TGAAAATAAG CTCAAGCAGA CAAATCTCCA GTGGATAAAG
       160        170        180        190        200
GTTAGACATT AACCATCTCT TCCGTCACAT GTGTTAAATG TTGCAAGTAT
       210        220        230        240        250
TTGTATGTAT TTTGTTTCCT GGGTGCTTCA TTGGTCGGGG AGGAGGCTGG
       260        270        280
TATGTGGATT GTTGTTTTGT TTTGTTTTTT-3'
```

In Table 8 is the exon and flanking intron sequences for the Exon located on a 1.6 Kb Hind III fragment from 512 to 622.

TABLE 8

```
5'      10         20         30         40         50
AAGCTTTGAT ACTGTGCTTT AAGTGTTTAC CCTTTGGAAA GAAAATAATT
        60         70         80         90        100
TTGACAGTGA TGTAGAAATA ATTATTTGAT ATTTATTTCA AAACAAAATT
       110        120        130        140        150
TATATCCAAT ACTAAACACA GAATTTTGTA AAACAATAAG TGTATAAAGT
       160        170        180        190        200
AAAATGAACA TTAGGATTAT TGAGATTATT GTAGCTAAAA CTAGTGTTTA
       210        220        230        240        250
TTCATATAAA TTATGTTAAT AAATTGTATT GTCATTATTG CATTTTACTT
       260        270        280        290        300
TTTTGAAAAG TAGTTAATGC CTGTGTTTCT ATATGAGTAT TATATAATTC
```

310	320	330	340	350
AAGAAGATAT	TGGATGAATT	TTTTTTTTAA	GTTTAATGTG	TTTCACATCT
360	370	380	390	400
CTGTTTCTTT	TCTCTGCACC	AAAAGTCACA	TTTTTGTGCC	CTTATGTACC
410	420	430	440	450
AGGCAGAAAT	TGATCTGCAA	TACATGTGGA	GTCTCCAAGG	GTATATTTAA
460	470	480	490	500
ATTTAGTAAT	TTTATTGCTA	ACTGTGAAGT	TAATCTGCAC	TATATGGGTT
510	520	530	540	550
CTTTTCCCCA	GGAAACTGAA	ATAGCAGTTC	AAGCTAAACA	ACCGGATGTG
560	570	580	590	600
GAAGAGATTT	TGTCTAAAGG	GCAGCATTTG	TACAAGGAAA	AACCAGCCAC
610	620	630	640	650
TCAGCCAGTG	AAGGTAATGA	AGCAACCTCT	AGCAATATCC	ATTACCTCAT
660	670	680	690	700
AATGGGTTAT	GCTTCGCCTG	TTGTACATTT	GCCATTGACG	TGGACTATTT
710	720	730	740	750
ATAATCAGTG	AAATAACTTG	TAAGGAAATA	CTGGCCATAC	TGTAATAGCA
760	770	780	790	800
GAGGCAAAGC	TGTCTTTTTG	ATCAGCATAT	CCTATTTATA	TATTGTGATC
810	820	830	840	
TTAAGGCTAT	TAACGAGTCA	TTGCTTTAAA	GGACTCATTT	CTGTC-3'

In Table 9 is the exon and flanking intron sequences for the Exon located on a 3.1 Kb Hind III fragment. The exon is from 519 to 751.

TABLE 9

5' 103	113	123	133	143
CCCATCTTGT	TTTGCCTTTG	TTTTTTCTTG	AATAAAAAAA	AAATAAGTAA
153	163	173	183	193
AATTTATTTC	CCTGGCAAGG	TCTGAAAACT	TTTGTTTTCT	TTACCACTTC
203	213	223	233	243
CACAATGTAT	ATGATTGTTA	CTGAGAAGGC	TTATTTAACT	TAAGTTACTT
253	263	273	283	293
GTCCAGGCAT	GAGAATGAGC	AAAATCGTTT	TTTAAAAAAT	TGTTAAATGT
303	313	323	333	343
ATATTAATGA	AAAGGTTGAA	TCTTTTCATT	TTCTACCATG	TATTGCTAAA
353	363	373	383	393
CAAAGTATCC	ACATTGTTAG	AAAAAGATAT	ATAATGTCAT	GAATAAGAGT
403	413	423	433	443
TTGGCTCAAA	TTGTTACTCT	TCAATTAAAT	TTGACTTATT	GTTATTGAAA
453	463	473	483	493
TTGGCTCTTT	AGCTTGTGTT	TCTAATTTTT	CTTTTTCTTC	TTTTTTCCTT
503	513	523	533	543
TTTGCAAAAA	CCCAAAATAT	TTTAGCTCCT	ACTCAGACTG	TTACTCTGGT
553	563	573	583	593
GACACAACCT	GTGGTTACTA	AGGAAACTGC	CATCTCCAAA	CTAGAAATGC
603	613	623	633	643
CATCTTCCTT	GATGTTGGAG	GTACCTGCTC	TGGCAGATTT	CAACCGGGCT
653	663	673	683	693
TGGACAGAAC	TTACCGACTG	GCTTTCTCTG	CTTGATCAAG	TTATAAAATC

703	713	723	733	743
ACAGAGGGTG	ATGGTGGGTG	ACCTTGAGGA	TATCAACGAG	ATGATCATCA
753	763	773	783	793
AGCAGAAGGT	ATGAGAAAAA	ATGATAAAAG	TTGGCAGAAG	TTTTTCTTTA
803	813	823	833	843
AAATGAAGAT	TTTCCACCAA	TCACTTTACT	CTCCTAGACC	ATTTCCCACC
853	863	873	883	893
AGTTCTTAGG	CAACTGTTTC	TCTCTCAGCA	AACACATTAC	TCTCACTATT
903	913	923	933	943
CAGCCTAAGT	ATAATCAGGT	ATAAATTAAT	GCAAATAACA	AAAGTAGCCA
953	963	973	983	993
TACATTAAAA	AGGAAAATAT	ACAAAAAAAA	AAAAAAAAAA	AAGCCAGAAA
1003	1013			
CCTACAGAAT	AGTGCTCTAG	TAATTAC-3'		

In Table 10 is the exon and flanking intron sequences for the Exon located on a 1.5 Kb Hind III fragment. The exon is from 190 to 337.

TABLE 10

5' 10	20	30	40	50
ATCTCTATCA	TTAGAGATCT	GAATATGAAA	TACTTGTCAA	AGTGAATGAA
60	70	80	90	100
AATTTNNTAA	ATTATGTATG	GTTAACATCT	TTAAATTGCT	TATTTTTAAA
110	120	130	140	150
TTGCCATGTT	TGTGTCCCAG	TTTGCATTAA	CAAATAGTTT	GAGAACTATG
160	170	180	190	200
TTGGAAAAAA	AAATAACAAT	TTTATTCTTC	TTTCTCCAGG	CTAGAAGAAC
210	220	230	240	250
AAAAGAATAT	CTTGTCAGAA	TTTCAAAGAG	ATTTAAATGA	ATTTGTTTTA
260	270	280	290	300
TGGTTGGAGG	AAGCAGATAA	CATTGCTAGT	ATCCCACTTG	AACCTGGAAA
310	320	330	340	350
AGAGCAGCAA	CTAAAAGAAA	AGCTTGAGCA	AGTCAAGGTA	ATTTTATTTT
360	370	380	390	400
CTCAAATCCC	CCAGGGCCTG	CTTGCATAAA	GAAGTATATG	AATCTATTTT
410	420	430	440	450
TTAATTCAAT	CATTGGTTTT	CTGCCCATTA	GGTTATTCAT	AGTTCCTTGC
460	470	480	490	500
TAAAGTGTTT	TTCTCACAAC	TTTATTTCTT	CTTAACCCTG	CAGTTCTGAA
510	520	530	540	550
CCAGTGCACA	TAAGAACATA	TGTATATATG	TGTGTGTGTG	TATTTATATA
560	570	580	590	600
TACACACACA	CATATTGCAT	CTATACATCT	ACACATATAG	ATGTATAGAT
610	620	630	640	650
TCAATATGTC	TAAAAATGTA	TATAATTCAC	AGTTTTTATC	TTTGATTTGA
660	670	680		
ATATTTAAGG	GACTGAGACT	CACACTCATA	TACTTTT-3'	

EXAMPLE 3

Prenatal Diagnosis and Detection of DMD Using PCR

An example of prenatal diagnosis with PCR deletion detection is demonstrated using synthesized oligonucleotide primers (set b, Table 1). This primer set corresponds to the intron sequences flanking exon 17 of the human DMD gene, a region which has been isolated and sequenced (Table 2).

The results of this analysis are shown in figure 3. The PCR products (one-twentieth of the total reaction) were obtained with template DNA isolated from a control male □, the male fetus being diagnosed Δ, the DMD carrier mother (O) and an affected male brother of the fetus ■. Also shown is a DNA molecular weight standard (MW: Hae III digested ϕX174 DNA). The results demonstrate that the affected male carries a deletion of exon 17, which was not amplified, but that the fetus does not carry the deletion and is therefore unaffected. These results indicate that PCR is useful in the diagnosis of DMD cases containing a deletion involving this exon.

EXAMPLE 4

Multiplex Detection

An example of multiplex detection is shown in Figures 3A and 3B.

This analysis was done using six primer pairs (sets a-f, Table 1) and the conditions described in Example 1. Automatic rather than manual amplification was performed. These oligonucleotide primers represent the flanking regions of six separate DMD gene exons. They were combined into a reaction vial and used for multiplex genomic DNA amplifications. Template DNA was isolated from lymphoblasts (from blood sample). Analysis was by agarose gel electrophoresis.

When non-deleted DNA was used as a template, the six dispersed regions of the DMD gene were simultaneously and specificially amplified (Figure 3A, Sample #534). Discrete deletions, which were detected with this method, are shown in Figures 3A and 3B. Several DNA samples containing normal, partial or total DMD gene deletions are shown. Figures 3A and 3B also show a DNA molecular weight standard (MW: Hae III digested ϕX174 DNA), and a negative control (-) where no template DNA was added to the reactions. Figure 3A also indicates which amplified DNA fragment corresponds to which exon (a-f) of Figure 1.

EXAMPLE 5

Prenatal Diagnosis

Multiplex PCR has been used successfully in several prenatal diagnoses. The conditions are as described above in Example 1. Figure 4 shows Multiplex DNA amplification for prenatal diagnosis of DMD. Shown are the results of amplification using DNA from affected males (AM: lymphoblast DNA) and male fetuses (MF: cultured amniotic fluid cell DNA) from six different families. Analysis was as described in Example 1. Both the affected male and the fetal DNA of DRL #s 521 and 531 display a deletion of region f (Figure 1). Thus these fetuses were diagnosed as affected. In DRL # 43C the affected male is deleted for all regions except f, while the fetus is unaffected. The affected male in DRL #483 is deleted for region a, while the male fetus is unaffected. Neither of the samples from DRL #s 485 or 469 displayed a deletion with this technique. Thus, if a deletion defect causes DMD in these families it occurred in an untested exon.

EXAMPLE 6

Prenatal diagnosis using multiplex DNA amplification of chorionic villus specimen (CVS) DNA

Figure 5 demonstrates Multiplex DNA amplification from CVS DNA. Both the affected male (AM; lymphoblast DNA) and the male fetus (MF; CVS DNA) from DRL # 92 display a deletion of regions e and f (Fig. 1). Thus the fetus was diagnosed as affected. CVS DNA from DRL # 120 did not display a deletion with this technique. Samples were analyzed as described in Example 1. These results demonstrate that the multiplex amplification technique works well for prenatal diagnosis when CVS DNA is used as the template for amplification.

EXAMPLE 7

Multiplex amplification of seven separate exons of the DMD gene

This example demonstrates that seven separate DNA sequences can be simultaneously amplified using the multiplex amplification technique. Conditions were as described in Example 1. Primer sets a-g (Table 1) were added to the reaction. Thus seven exon regions of the DMD gene (Figure 1) were amplified (Figure 6).

EXAMPLE 8

Multiplex DNA amplification for the simultaneous detection of mutations leading to multiple common genetic diseases

This example describes how the multiplex amplification technique can be used to simultaneously screen a newborn male for any of the most common mutations leading to DMD, sickle-cell anemia and α_1-antitrypsin deficiency. In this assay any or all of the primers sets listed in Table 1 can be used for multiplex DNA amplification to diagnose the majority of possible DMD gene deletions. Additionally, primer sets can be added to the amplification reaction to identify mutations leading to additional genetic diseases. Other primer sets include:

A.

5′-TGGTCTCCTTAAACCTGTCTT-3′

5′-ACACAACTGTGTTCACTAG-3′

These oligonucleotides amplify a 167 bp segment of the human β-globin gene, containing the DNA base that is mutated in β^s (sickle-cell) hemoglobinopathy. The presence or absence of the mutant β^s sequence is then determined either by separate dot blot or Southern blot hybridization of the multiplex amplification reaction with each of two labelled allele-specific oligonucleotide (ASO) probes specific for the normal or β^s sequence. The sequence of these two ASO probes is:

1) Normal: 5′-CTCCTGAGGAGA-3′

2) β^s: 5′-CTCCTGTGGAGA-3′

If dot blot hybridization is used, a separate application of DNA from the multiplex amplification reaction to a DNA membrane, such as nitrocellulose, is required for each probe that will be used in the hybridization. Hybridization of each labelled probe, whether the probes are complementary to individual alleles of a given gene or to separate genes, must be performed individually. Alternatively and preferably, two aliquots of the amplification reaction are separately electrophoresed on agarose gels and transferred to nitrocellulose or a similar membrane using Southern analysis. Each of the two Southern blots are then hybridized with one member of each labelled set of specific ASO primers. Thus each known mutant or normal allele of each DNA fragment amplified in the multiplex reaction can be determined.

In addition to the above described primer sets the following oligonucleotide primers can also be added to the amplification procedure:

B.

5'-ACGTGGAGTGACGATGCTCTTCCC-3'

5'-GTGGGATTCACCACTTTTCCC-3'

These primers produce a 450 bp DNA fragment containing the DNA base change that produces the Z allele of the α_1-antitrypsin gene and leads to α_1-antitrypsin deficiency. The Z allele and the normal M allele are distinguished from other alleles in the multiplex amplification reaction by hybridization with the ASO probes:

1) Normal (M)allele:5'-ATCGACGAGAAA-3'

2) Mutant (Z)allele:5'-ATCGACAAGAAA-3'

Hybridization analysis is performed in parallel with the β-globin probes as described above.

In addition, the oligonucleotides

C.

5'-GAAGTCAAGGACACCGAGGAA-3'

5'-AGCCCTCTGGCCAGTCCTAGTG-3'

can also be added to the multiplex reaction to produce a 340 bp DNA region of the α_1-antitrypsin gene that contains the DNA base change that produces the S allele and leads to α_1-antitrypsin deficiency. The S allele is distinguished from other alleles in the multiplex amplification as described above for the β^s and Z alleles by using the two ASO probes specific for the M and S allele:

Normal (M)allele 5'-ACCTGGAAAATG-3'

Mutant (S)allele 5'-ACCTGGTAAATG-3'

Using the primers described in Table 1 and in A, B and C of this example the common mutations leading to DMD, sickle cell anemia and α_1-antitrypsin deficiency can be simultaneously determined.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well, those inherent therein. The methods procedures and techniques described herein are presently representative of the preferred embodiments, are intended to be exemplary, and are not intended as limitations on the scope. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention or defined by the scope of the appended claims.

Claims

1. A method for simultaneously detecting deletions at a plurality of DNA sequences, comprising the steps of:
treating said DNA to form single-stranded complementary strands;
adding a plurality of paired oligonucleotide primers, each pair specific for a different sequence, one primer of each pair substantially complementary to a part of the sequence in the sense-strand and the other primer of each pair substantially complementary to a different part of the same sequence in the complementary anti-sense strand;
annealing the plurality of primers to their complementary sequences;
simultaneously extending said plurality of annealed primers from each primer's 3' terminus to synthesize an extension product complementary to the strands annealed to each primer, said extension products, after separation from their complement, serving as templates for the synthesis of an extension product from the other primer of each pair;
separating said extension products from said templates to produce single-stranded molecules;
amplifying said single stranded molecules by repeating, at least once, said annealing, extending and separating steps; and
identifying said amplified extension products from each different sequence.

2. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein said sequences are selected from the group of sequences on the X and Y chromosomes.

3. The method of Claim 2 for the detection of X-linked disease, wherein said genomic DNA sequences contain a deletion that causes a genetic disease.

4. The method of Claim 3 for the detection of said X-linked genetic diseases selected from the group consisting of ornithine transcarbamylase deficiency, hypoxanthine phosphoribosyltransferfase deficiency, steroid sulfatase deficiency and X-linked muscular dystrophy.

5. The method of Claim 4 for the detection of X-linked muscular dystrophy, wherein said plurality of paired primers are complementary to different sequences within the gene coding for the dystrophin protein.

6. The method of Claim 5, wherein the plurality of paired primers is selected from the group consisting of:

(1) 5'-GACTTTCGATGTTGAGATTACTTTCCC-3'
(2) 5'-AAGCTTGAGATGCTCTCACCTTTTCC-3',
(1) 5'-GTCCTTTACACACTTTACCTGTTGAG-3'
(2) 5'-GGCCTCATTCTCATGTTCTAATTAG-3',
(1) 5'-AAACATGGAACATCCTTGTGGGGAC-3'
(2) 5'-CATTCCTATTAGATCTGTCGCCCTAC-3',
(1) 5'-GATAGTGGGCTTTACTTACATCCTTC-3'
(2) 5'-GAAAGCACGCAACATAAGATACACCT-3',
(1) 5'-CTTGATCCATATGCTTTTACCTGCA-3'
(2) 5'-TCCATCACCCTTCAGAACCTGATCT-3',
(1) 5'-GAATACATTGGTTAAATCCCAACATG-3'
(2) 5'-CCTGAATAAAGTCTTCCTTACCACAC-3', and
(1) 5'-TTCTACCACATCCCATTTTCTTCCA-3'
(2) 5'-GATGGCAAAAGTGTTGAGAAAAAGTC-3'.

7. The method of Claim 3, wherein said genomic DNA is from fetal tissue.

8. The method of Claim 1 for detecting deletions at a plurality of genomic DNA sequences, wherein the plurality of paired primers is selected from the group consisting of:

(1) 5'-GACTTTCGATGTTGAGATTACTTTCCC-3'
(2) 5'-AAGCTTGAGATGCTCTCACCTTTTCC-3',
(1) 5'-GTCCTTTACACACTTTACCTGTTGAG-3'
(2) 5'-GGCCTCATTCTCATGTTCTAATTAG-3',
(1) 5'-AAACATGGAACATCCTTGTGGGGAC-3'
(2) 5'-CATTCCTATTAGATCTGTCGCCCTAC-3',
(1) 5'-GATAGTGGGCTTTACTTACATCCTTC-3'
(2) 5'-GAAAGCACGCAACATAAGATACACCT-3',
(1) 5'-CTTGATCCATATGCTTTTACCTGCA-3'
(2) 5'-TCCATCACCCTTCAGAACCTGATCT-3',
(1) 5'-GAATACATTGGTTAAATCCCAACATG-3'
(2) 5'-CCTGAATAAAGTCTTCCTTACCACAC-3',
(1) 5'-TTCTACCACATCCCATTTTCTTCCA-3'
(2) 5'-GATGGCAAAAGTGTTGAGAAAAAGTC-3',
(1) 5'-TGGTCTCCTTAAACCTGTCTT-3'
(2) 5'-ACACAACTGTGTTCACTAG-3',
(1) 5'-ACGTGGAGTGACGATGCTCTTCCC-3'
(2) 5'-GTGGGATTCACCACTTTTCCC-3', and
(1) 5'-GAAGTCAAGGACACCGAGGAA-3'
(2) 5'-AGCCCTCTGGCCAGTCCTAGTG-3'.

9. A DNA sequence of the formula:

5' 10	20	30	40	50
TAAATTGACT	TTCGATGTTG	AGATTACTTT	CCCTTGCTAT	TTCAGTGAAC
60	70	80	90	100
CAAACTTAAG	TCAGATAAAA	CAATTTTATT	TGGCTTCAAT	ATGGTGCTAT
110	120	130	140	150
TTTGATCTGA	AGGTCAATCT	ACCAACAAGC	AAGAACAGTT	TCTCATTATT
160	170	180	190	200
TTCCTTTGCC	ACTCCAAGCA	GTCTTTACTG	AAGTCTTTCG	AGCAATGTCT
210	220	230	240	250
GACCTCTGTT	TCAATACTTC	TCACAGATTT	CACAGGCTGT	CACCACCACT
260	270	280	290	300
CAGCCATCAC	TAACACAGAC	AACTGTAATG	GAAACAGTAA	CTACGGTGAC

310	320	330	340	350
CACAAGGGAA	CAGATCCTGG	TAAAGCATGC	TCAAGAGGAA	CTTCCACCAC
360	370	380	390	400
CACCTCCCCA	AAAGAAGAGG	CAGATTACTG	TGGATTCTGA	AATTAGGAAA
410	420	430	440	450
AGGTGAGAGC	ATCTCAAGCT	TTTATCTGCA	AATGAAGTGG	AGAAAACTCA
460	470	480	490	500
TTTACAGCAG	TTTTGTTGGT	GGTGTTTTCA	CTTCAGCAAT	ATTTCCAGAA
510	520	530	540	550
TCCTCGGGTA	CCTGTAATGT	CAGTTAATGT	AGTGAGAAAA	ATTATGAAGT
560	570	580	590	600
ACATTTTAAA	ACTTTCACAA	GAAATCACTA	TCGCAACAGA	AACTAAATGC
610	620	630	640	650
TTAATGGAAA	TGGTGTTTTC	TGGGGTGAAA	GAAGAAACTA	TAGAAACTAT
660	670	680	690	700
AGGTGATAAA	CTACTGTGGT	AGCATTTTAA	TCCTAAAAGT	TTCTTTCTTT
710	720	730	740	750
CTTTTTTTTT	TTTCTTCCTT	ATAAAGGGCC	TGCTTGTTGA	GTCCCTAGTT
760	770	780	790	800
TTGCATTAAA	TGTCTTTTTT	TTCCAGTAAC	GGAAAGTGCA	TTTTCATGAA
810	820	830	840	850
GAAGTACACC	TATAATAGAT	GGGATCCATC	CTGGTAGTTT	ACGAGAACAT
860	870	880	890	900
GATGTCTCAG	TCTGCGCATC	CTAAATCAGG	AGTAATTACA	GAACACATTT
910	920	930	940	950
CCTGTTCTTT	GATATTTATA	AAGTCTTATC	TTGAAGGTGT	TAGAATTTTT
960	970	980	990	1000
AACTGATCTT	TTTGTGACTA	TTCAGAATTA	TGCATTTTAG	ATAAGATTAG
1010	1020	1030	1040	
GTATTATGTA	AATCAGTGGA	TATATTAAAT	GATGGCAATA	A-3'

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

10. A DNA sequence of the formula:

5' 10	20	30	40	50
TGTCCAAAAT	AGTTGACTTT	CTTTCTTTAA	TCAATAAATA	TATTACTTTA
60	70	80	90	100
AAGGGAAAAA	TTGCAACCTT	CCATTTAAAA	TCAGCTTTAT	ATTGAGTATT
110	120	130	140	150
TTTTTAAAAT	GTTGTGTGTA	CATGCTAGGT	GTGTATATTA	ATTTTTATTT
160	170	180	190	200
GTTACTTGAA	ACTAAACTCT	GCAAATGCAG	GAAACTATCA	GAGTGATATC
210	220	230	240	250
TTTGTCAGTA	TAACCAAAAA	ATATACGCTA	TATCTCTATA	ATCTGTTTTA
260	270	280	290	300
CATAATCCAT	CTATTTTTCT	TGATCCATAT	GCTTTTACCT	GCAGGCGATT

```
       310         320         330         340         350
TGACAGATCT  GTTGAGAAAT  GGCGGCGTTT  TCATTATGAT  ATAAAGATAT
       360         370         380         390         400
TTAATCAGTG  GCTAACAGAA  GCTGAACAGT  TTCTCAGAAA  GACACAAATT
       410         420         430         440         450
CCTGAGAATT  GGGAACATGC  TAAATACAAA  TGGTATCTTA  AGGTAAGTCT
       460         470         480         490         500
TTGATTTGTT  TTTTCGAAAT  TGTATTTATC  TTCAGCACAT  CTGGACTCTT
       510         520         530         540         550
TAACTTCTTA  AAGATCAGGT  TCTGAAGGGT  GATGGAAATT  ACTTTTGACT
       560         570         580
GTTGTTGTCA  TCATTATATT  ACTAGAAAGA  AAA-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

11. A DNA sequence of the formula:

```
5'      10          20          30          40          50
ACCCAAATAC  TTTGTTCATG  TTTAAATTTT  ACAACATTTC  ATAGACTATT
        60          70          80          90         100
AAACATGGAA  CATCCTTGTG  GGGACAAGAA  ATCGAATTTG  CTCTTGAAAA
       110         120         130         140         150
GGTTTCCAAC  TAATTGATTT  GTAGGACATT  ATAACATCCT  CTAGCTGACA
       160         170         180         190         200
AGCTTACAAA  AATAAAAACT  GGAGCTAACC  GAGAGGGTGC  TTTTTTCCCT
       210         220         230         240         250
GACACATAAA  AGGTGTCTTT  CTGTCTTGTA  TCCTTTGGAT  ATGGGCATGT
       260         270         280         290         300
CAGTTTCATA  GGGAAATTTT  CACATGGAGC  TTTTGTATTT  CTTTCTTTGC
       310         320         330         340         350
CAGTACAACT  GCATGTGGTA  GCACACTGTT  TAATCTTTTC  TCAAATAAAA
       360         370         380         390         400
AGACATGGGG  CTTCATTTTT  GTTTTGCCTT  TTTGGTATCT  TACAGGAACT
       410         420         430         440         450
CCAGGATGGC  ATTGGGCAGC  GGCAAACTGT  TGTCAGAACA  TTGAATGCAA
       460         470         480         490         500
CTGGGGAAGA  AATAATTCAG  CAATCCTCAA  AAACAGATGC  CAGTATTCTA
       510         520         530         540         550
CAGGAAAAAT  TGGGAAGCCT  GAATCTGCGG  TGGCAGGAGG  TCTGCAAACA
       560         570         580         590         600
GCTGTCAGAC  AGAAAAAAGA  GGTAGGGCGA  CAGATCTAAT  AGGAATGAAA
       610         620
ACATTTTAGC AGACTTTTTA AGCTT-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

12. A DNA sequence of the formula:

5' 10	20	30	40	50
TTTTGTAGAC	GGTTAATGAA	TAATTTTGAA	TACATTGGTT	AAATCCCAAC
60	70	80	90	100
ATGTAATATA	TGTAAATAAT	CAATATTATG	CTGCTAAAAT	AACACAAATC
110	120	130	140	150
AGTAAGATTC	TGTAATATTT	CATGATAAAT	AACTTTTGAA	AATATATTTT
160	170	180	190	200
TAAACATTTT	GCTTATGCCT	TGAGAATTAT	TTACCTTTTT	AAAATGTATT
210	220	230	240	250
TTCCTTTCAG	GTTTCCAGAG	CTTTACCTGA	GAAACAAGGA	GAAATTGAAG
260	270	280	290	300
CTCAAATAAA	AGACCTTGGG	CAGCTTGAAA	AAAAGCTTGA	AGACCTTGAA
310	320	330	340	350
GAGCAGTTAA	ATCATCTGCT	GCTGTGGTTA	TCTCCTATTA	GGAATCAGTT
360	370	380	390	400
GGAAATTTAT	AACCAACCAA	ACCAAGAAGG	ACCATTTGAC	GTTAAGGTAG
410	420	430	440	450
GGGAACTTTT	TGCTTTAATA	TTTTTGTCTT	TTTTAAGAAA	AATGGCAATA
460	470	480	490	500
TCACTGAATT	TTCTCATTTG	GTATCATTAT	TAAAGACAAA	ATATTACTTG
510	520	530	540	550
TTAAAGTGTG	GTAAGGAAGA	CTTTATTCAG	GATAACCACA	ATAGGCACAG
560	570	580	590	600
GGACCACTGC	AATGGAGTAT	TACAGGAGGT	TGGATAGAGA	GAGATTGGGC
610	620	630	640	650
TCAACTCTAA	ATACAGCACA	GTGGAAGTAG	GAATTTATAG	C-3'

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

13. A DNA sequence of the formula:

5' 10	20	30	40	50
TGAGAAATAA	TAGTTCCGGG	GTGACTGATA	GTGGGCTTTA	CTTACATCCT
60	70	80	90	100
TCTCAATGTC	CAATAGATGC	CCCCAAATGC	GAACATTCCA	TATATTATAA
110	120	130	140	150
ATTCTATTGT	TTTACATTGT	GATGTTCAGT	AATAAGTTGC	TTTCAAAGAG
160	170	180	190	200
GTCATAATAG	GCTTCTTTCA	AATTTTCAGT	TTACATAGAG	TTTTAATGGA
210	220	230	240	250
TCTCCAGAAT	CAGAAACTGA	AAGAGTTGAA	TGACTGGCTA	ACAAAACAGA

260	270	280	290	300
AGAAAGAACA	AGGAAAATGG	AGGAAGAGCC	TCTTGGACCT	GATCTTGAAG
310	320	330	340	350
ACCTAAAACG	CCAAGTACAA	CAACATAAGG	TAGGTGTATC	TTATGTTGCG
360	370	380	390	400
TGCTTTCTAC	TAGAAAGCAA	ACTCTGTGTA	TAGTACCTAT	ACACAGTAAC
410	420	430	440	450
ACAGATGACA	TGGTTGATGG	GAGAGAATTA	AAACTTAAAG	TCAGCCATAT
460	470	480	490	500
TTTAAAAATT	ATTTTTACCT	AATTGTTTTT	GCAATCTTTG	TTGCCAATGG
510	520	530	540	550
CCTTGAATAA	GTCCCCTCCA	AAATTCAGGT	GATTGTATTA	GGAGATGGAA
560	570	580	590	600
TATTTAAGGG	TGAATAATCC	ATCAGGGCTC	CTCCCTTAAG	AATAGGATCA
610	620	630	640	650
AGTCCCATAT	AAAAGAGGCT	TCACACAGTG	TTCTCCTATC	TCTTGACCCT
660	670	680	690	700
CCACCATGCA	CCACCATGTG	AAAACTCTGT	GAAAAGGCCC	TCACCAGATG
710	720	730	740	750
CTAACATCTT	GATCTTGGAT	TTCCCAAACT	CGAGAACTGT	GAAAAAATAA
760	770	780	790	800
AGGTACATTC	TTCCTAAATT	ACCTCATTCT	CATTTAAACA	CACAAAGTGC
810				
ACACATAGCT	G-3'			

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

14. A DNA sequence of the formula:

5' 10	20	30	40	50
TTACTGGTGG	AAGAGTTGCC	CCTGCGCCAG	GGAATTCTCA	AACAATTAAA
60	70	80	90	100
TGAAACTGGA	GGACCCGTGC	TTGTAAGTGC	TCCCATAAGC	CCAGAAGAGC
110	120	130	140	150
AAGATAAACT	TGAAAATAAG	CTCAAGCAGA	CAAATCTCCA	GTGGATAAAG
160	170	180	190	200
GTTAGACATT	AACCATCTCT	TCCGTCACAT	GTGTTAAATG	TTGCAAGTAT
210	220	230	240	250
TTGTATGTAT	TTTGTTTCCT	GGGTGCTTCA	TTGGTCGGGG	AGGAGGCTGG
260	270	280		
TATGTGGATT	GTTGTTTTGT	TTTGTTTTTT-3'		

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

15. A DNA sequence of the formula:

5' 10	20	30	40	50
AAGCTTTGAT	ACTGTGCTTT	AAGTGTTTAC	CCTTTGGAAA	GAAAATAATT
60	70	80	90	100
TTGACAGTGA	TGTAGAAATA	ATTATTTGAT	ATTTATTTCA	AAACAAAATT
110	120	130	140	150
TATATCCAAT	ACTAAACACA	GAATTTTGTA	AAACAATAAG	TGTATAAAGT
160	170	180	190	200
AAAATGAACA	TTAGGATTAT	TGAGATTATT	GTAGCTAAAA	CTAGTGTTTA
210	220	230	240	250
TTCATATAAA	TTATGTTAAT	AAATTGTATT	GTCATTATTG	CATTTTACTT
260	270	280	290	300
TTTTGAAAAG	TAGTTAATGC	CTGTGTTTCT	ATATGAGTAT	TATATAATTC
310	320	330	340	350
AAGAAGATAT	TGGATGAATT	TTTTTTTTAA	GTTTAATGTG	TTTCACATCT
360	370	380	390	400
CTGTTTCTTT	TCTCTGCACC	AAAAGTCACA	TTTTTGTGCC	CTTATGTACC
410	420	430	440	450
AGGCAGAAAT	TGATCTGCAA	TACATGTGGA	GTCTCCAAGG	GTATATTTAA
460	470	480	490	500
ATTTAGTAAT	TTTATTGCTA	ACTGTGAAGT	TAATCTGCAC	TATATGGGTT
510	520	530	540	550
CTTTTCCCCA	GGAAACTGAA	ATAGCAGTTC	AAGCTAAACA	ACCGGATGTG
560	570	580	590	600
GAAGAGATTT	TGTCTAAAGG	GCAGCATTTG	TACAAGGAAA	AACCAGCCAC
610	620	630	640	650
TCAGCCAGTG	AAGGTAATGA	AGCAACCTCT	AGCAATATCC	ATTACCTCAT
660	670	680	690	700
AATGGGTTAT	GCTTCGCCTG	TTGTACATTT	GCCATTGACG	TGGACTATTT
710	720	730	740	750
ATAATCAGTG	AAATAACTTG	TAAGGAAATA	CTGGCCATAC	TGTAATAGCA
760	770	780	790	800
GAGGCAAAGC	TGTCTTTTTG	ATCAGCATAT	CCTATTTATA	TATTGTGATC
810	820	830	840	
TTAAGGCTAT	TAACGAGTCA	TTGCTTTAAA	GGACTCATTT	CTGTC-3'

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

16. A DNA sequence of the formula:

5' 103	113	123	133	143
CCCATCTTGT	TTTGCCTTTG	TTTTTTCTTG	AATAAAAAAA	AAATAAGTAA
153	163	173	183	193
AATTTATTTC	CCTGGCAAGG	TCTGAAAACT	TTTGTTTTCT	TTACCACTTC

203	213	223	233	243
CACAATGTAT	ATGATTGTTA	CTGAGAAGGC	TTATTTAACT	TAAGTTACTT
253	263	273	283	293
GTCCAGGCAT	GAGAATGAGC	AAAATCGTTT	TTTAAAAAAT	TGTTAAATGT
303	313	323	333	343
ATATTAATGA	AAAGGTTGAA	TCTTTTCATT	TTCTACCATG	TATTGCTAAA
353	363	373	383	393
CAAAGTATCC	ACATTGTTAG	AAAAAGATAT	ATAATGTCAT	GAATAAGAGT
403	413	423	433	443
TTGGCTCAAA	TTGTTACTCT	TCAATTAAAT	TTGACTTATT	GTTATTGAAA
453	463	473	483	493
TTGGCTCTTT	AGCTTGTGTT	TCTAATTTTT	CTTTTTCTTC	TTTTTTCCTT
503	513	523	533	543
TTTGCAAAAA	CCCAAAATAT	TTTAGCTCCT	ACTCAGACTG	TTACTCTGGT
553	563	573	583	593
GACACAACCT	GTGGTTACTA	AGGAAACTGC	CATCTCCAAA	CTAGAAATGC
603	613	623	633	643
CATCTTCCTT	GATGTTGGAG	GTACCTGCTC	TGGCAGATTT	CAACCGGGCT
653	663	673	683	693
TGGACAGAAC	TTACCGACTG	GCTTTCTCTG	CTTGATCAAG	TTATAAAATC
703	713	723	733	743
ACAGAGGGTG	ATGGTGGGTG	ACCTTGAGGA	TATCAACGAG	ATGATCATCA
753	763	773	783	793
AGCAGAAGGT	ATGAGAAAAA	ATGATAAAAG	TTGGCAGAAG	TTTTTCTTTA
803	813	823	833	843
AAATGAAGAT	TTTCCACCAA	TCACTTTACT	CTCCTAGACC	ATTTCCCACC
853	863	873	883	893
AGTTCTTAGG	CAACTGTTTC	TCTCTCAGCA	AACACATTAC	TCTCACTATT
903	913	923	933	943
CAGCCTAAGT	ATAATCAGGT	ATAAATTAAT	GCAAATAACA	AAAGTAGCCA
953	963	973	983	993
TACATTAAAA	AGGAAAATAT	ACAAAAAAAA	AAAAAAAAAA	AAGCCAGAAA
1003	1013			
CCTACAGAAT	AGTGCTCTAG	TAATTAC 3'		

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

17. A DNA sequence of the formula:

5' 10	20	30	40	50
ATCTCTATCA	TTAGAGATCT	GAATATGAAA	TACTTGTCAA	AGTGAATGAA
60	70	80	90	100
AATTTNNTAA	ATTATGTATG	GTTAACATCT	TTAAATTGCT	TATTTTTAAA
110	120	130	140	150
TTGCCATGTT	TGTGTCCCAG	TTTGCATTAA	CAAATAGTTT	GAGAACTATG
160	170	180	190	200
TTGGAAAAAA	AAATAACAAT	TTTATTCTTC	TTTCTCCAGG	CTAGAAGAAC

```
       210        220        230        240        250
AAAAGAATAT CTTGTCAGAA TTTCAAAGAG ATTTAAATGA ATTTGTTTTA
       260        270        280        290        300
TGGTTGGAGG AAGCAGATAA CATTGCTAGT ATCCCACTTG AACCTGGAAA
       310        320        330        340        350
AGAGCAGCAA CTAAAAGAAA AGCTTGAGCA AGTCAAGGTA ATTTTATTTT
       360        370        380        390        400
CTCAAATCCC CCAGGGCCTG CTTGCATAAA GAAGTATATG AATCTATTTT
       410        420        430        440        450
TTAATTCAAT CATTGGTTTT CTGCCCATTA GGTTATTCAT AGTTCCTTGC
       460        470        480        490        500
TAAAGTGTTT TTCTCACAAC TTTATTTCTT CTTAACCCTG CAGTTCTGAA
       510        520        530        540        550
CCAGTGCACA TAAGAACATA TGTATATATG TGTGTGTGTG TATTTATATA
       560        570        580        590        600
TACACACACA CATATTGCAT CTATACATCT ACACATATAG ATGTATAGAT
       610        620        630        640        650
TCAATATGTC TAAAAATGTA TATAATTCAC AGTTTTTATC TTTGATTTGA
       660        670        680
ATATTTAAGG GACTGAGACT CACACTCATA TACTTTT-3'
```

and fragments and derivatives thereof, said fragments and derivatives complementary to the sense and anti-sense strands of the gene coding for dystrophin, said fragments and derivatives capable of annealing to said strands of the dystrophin gene and amplifying dystrophin sequences.

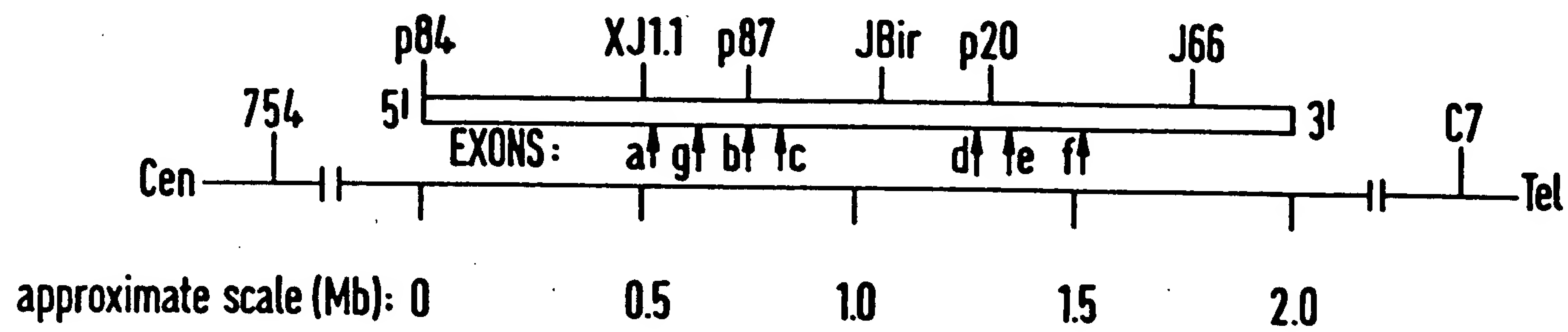
p84
XJ1.1
p87
JBir
p20
J66
754
5'
3'
C7
EXONS:
a
g
b
c
d
e
f
Cen
Tel
approximate scale (Mb): 0
0.5
1.0
1.5
2.0

FIG.1

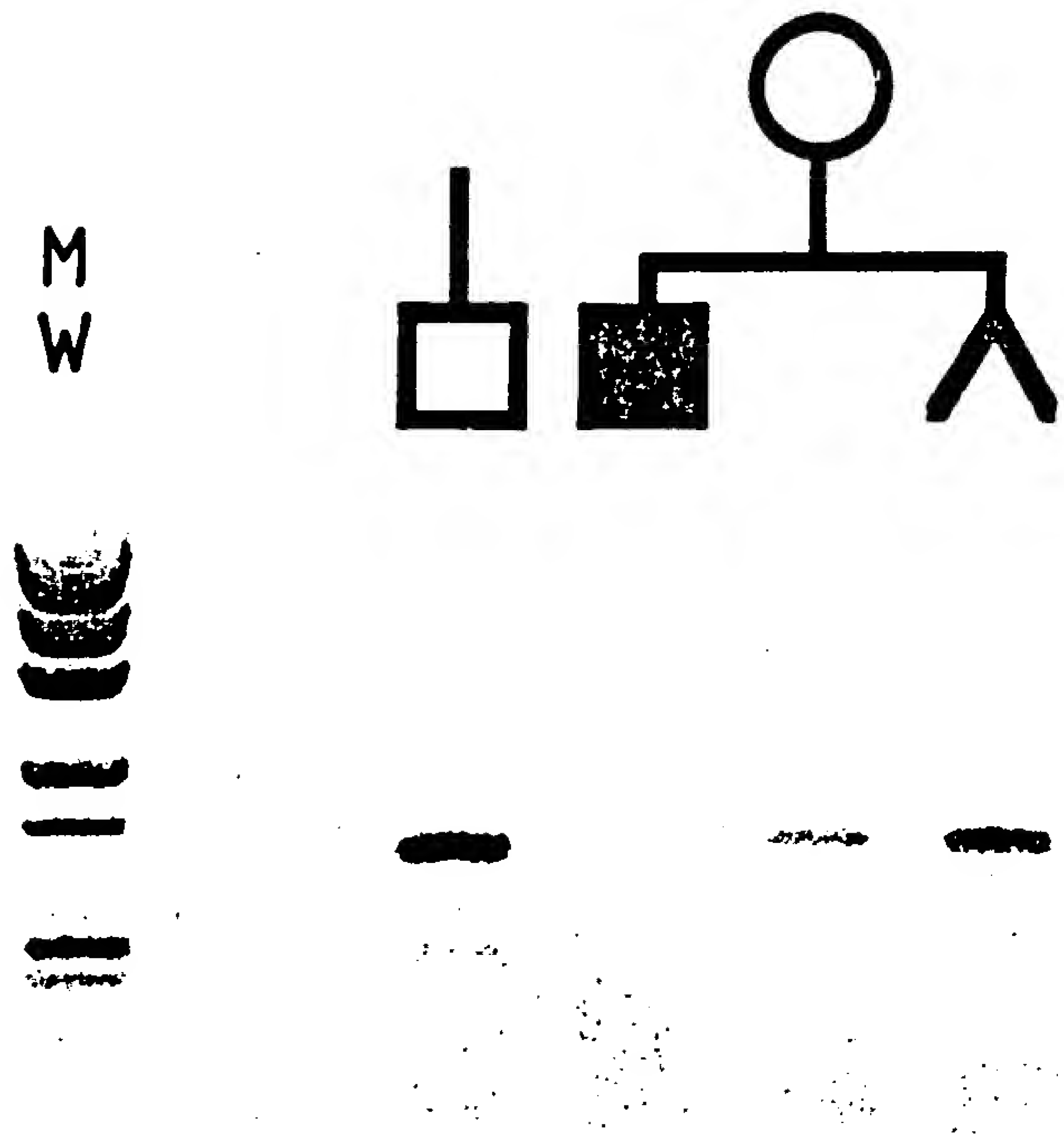

FIG.2

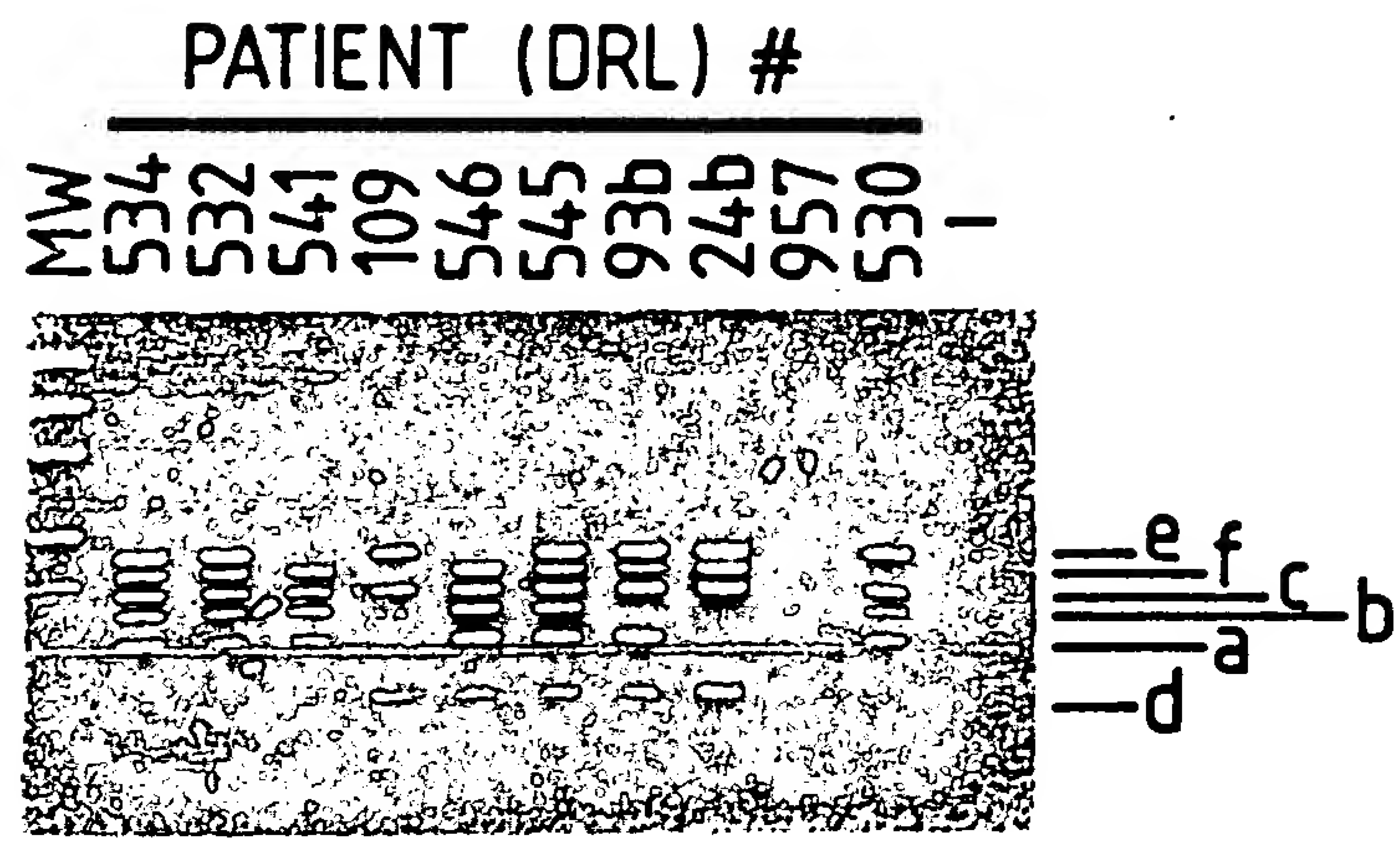
PATIENT (DRL) #
MW 534 532 541 109 546 545 93b 24b 957 530 I
e f c b a d

FIG.3A

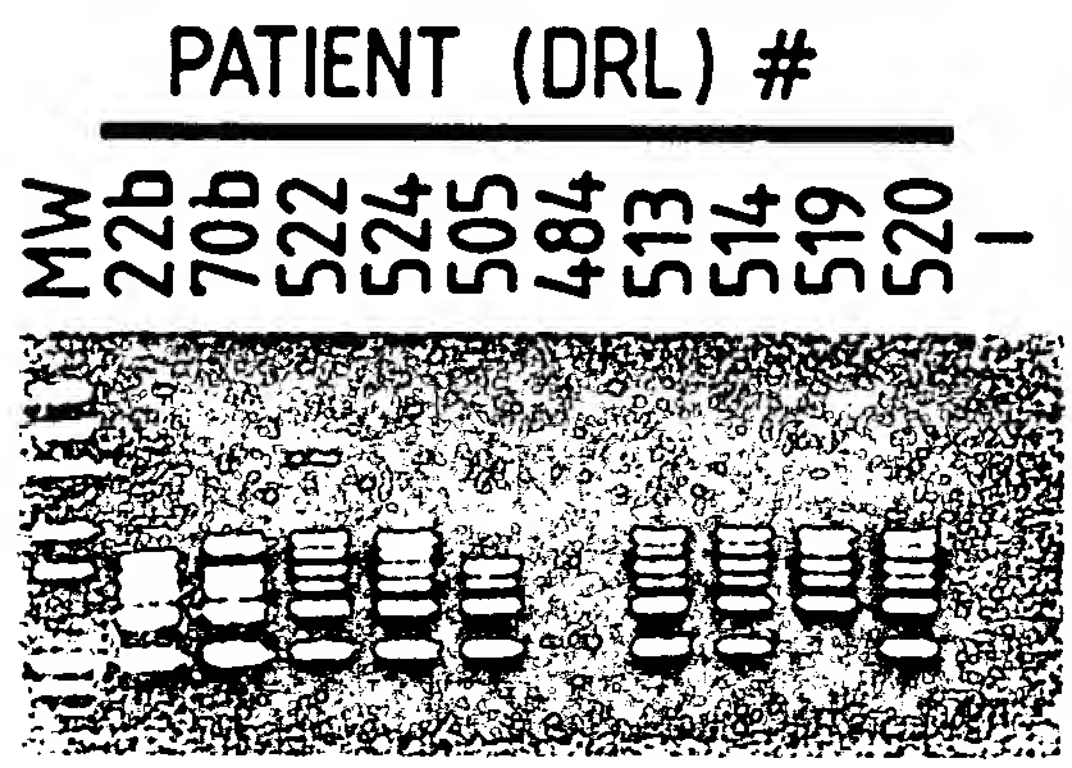
PATIENT (DRL) #
MW 22b 70b 522 524 505 484 513 514 519 520 I

FIG.3B

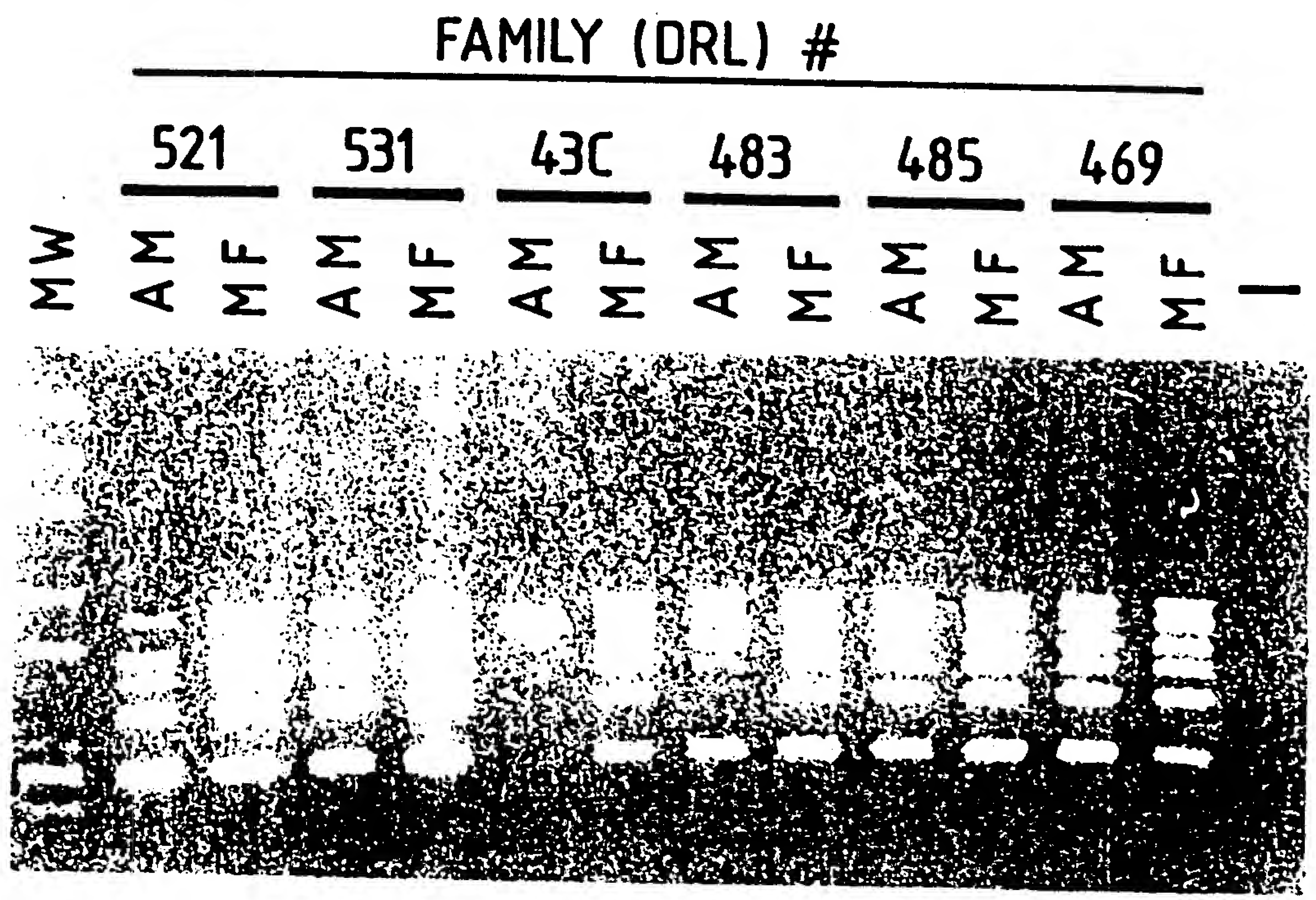
FAMILY (DRL) #
521
531
43C
483
485
469
MW
AM
MF
AM
MF
AM
MF
AM
MF
AM
MF
AM
MF
–

FIG.4

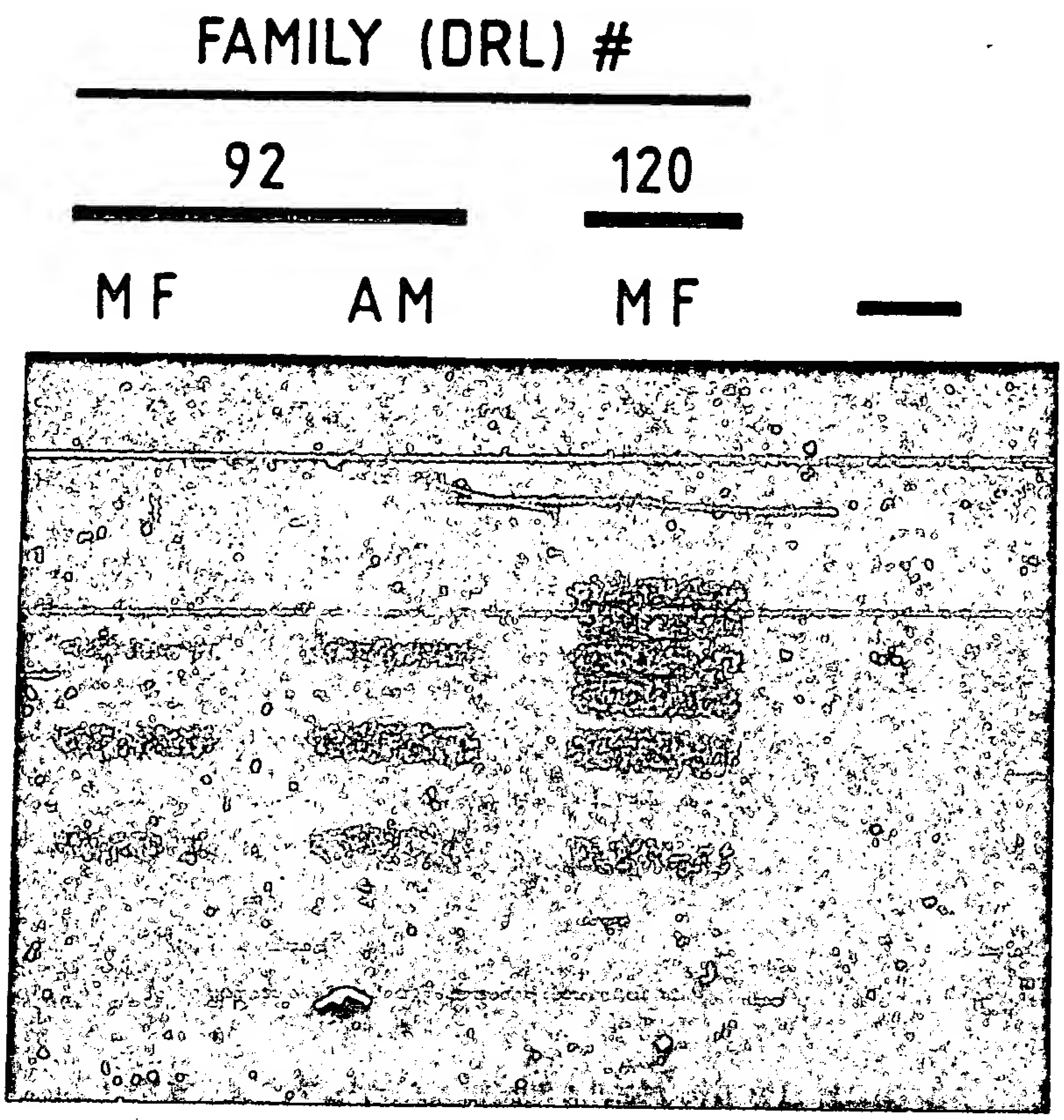

FIG.5

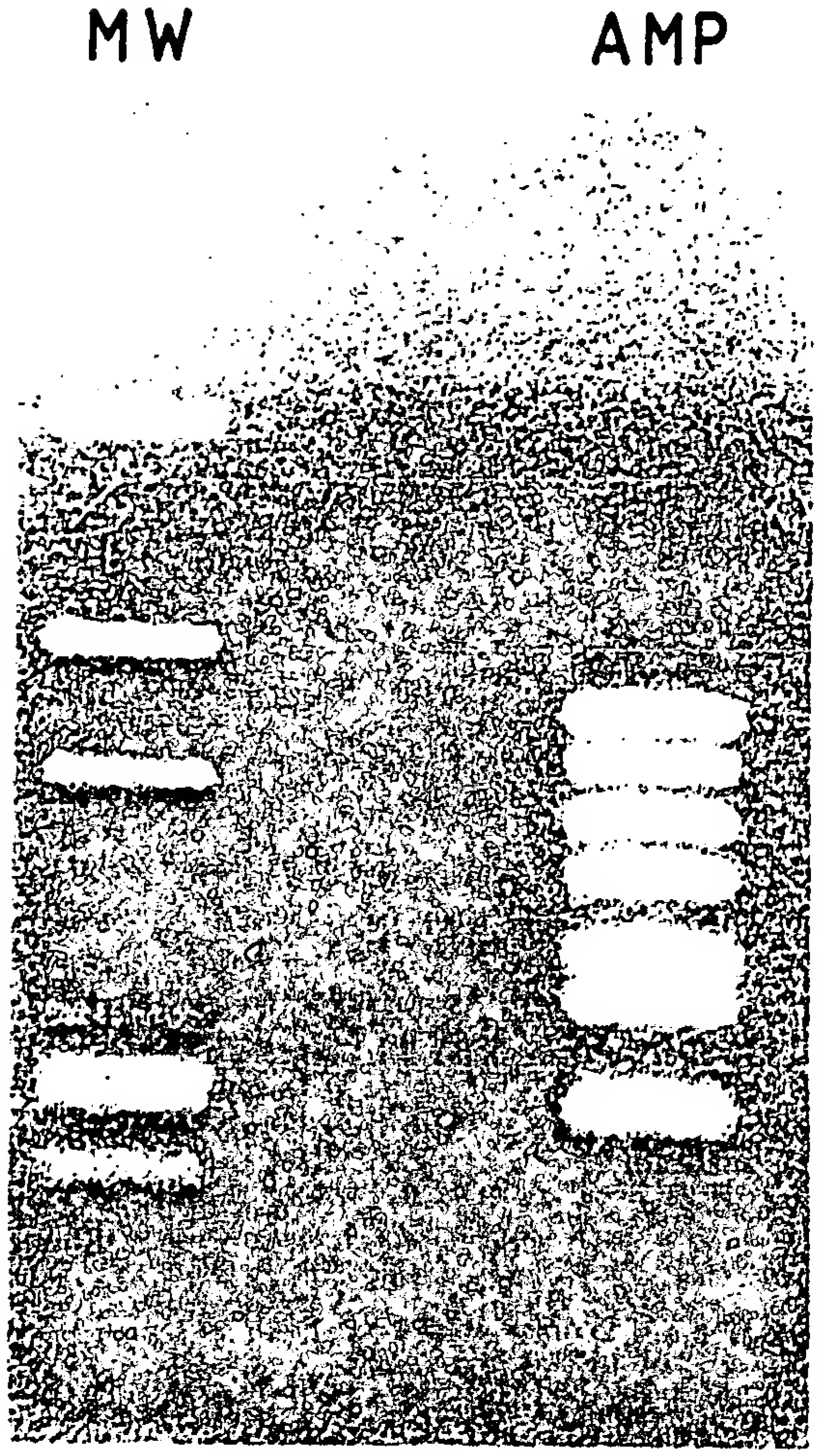

FIG.6